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(71) Applicant: SMITHKLINE BIOLOGICALS S.A.
Rue du Tilleul, 13
B-1320 Genval Rixensart (BE)

(72) Inventor: Capiau, Carine
Rue Astrid Harveng, 5
B-7092 Mons (BE)

Locht, Camille
Avenue Bel Horizon, 40
B-1320 Genval (Rixensart) (BE)

(74) Representative: Tasset, Gérard
Smithkline Biologicals s.a. Rue de l'Institut 89
B-1330 Rixensart (BE)

(54) Bordetella pertussis vaccine.

(57) A vaccine for protection against whooping cough which comprises protein encoded by a B. pertussis toxin amino acid coding sequence containing an entire or truncated B. pertussis toxin S1 subunit amino acid coding sequence which contains a modification of the tryptophan residue at amino acid position 26.

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Description

BORDETELLA PERTUSSIS VACCINE

Field of the Invention

This invention relates to a vaccine for protection against whooping cough which comprises protein encoded by a B. pertussis toxin coding sequence containing an entire or truncated B. pertussis toxin S1 subunit amino acid coding sequence which contains a modification of the tryptophan residue at amino acid position 26.

Background of the Invention

Whooping Cough, or pertussis, is a highly infectious disease which primarily affects children. In addition to causing respiratory complications, whooping cough may result in nerve damage and high mortality, particularly in children in low socioeconomic groups and in newborn infants without maternal anti-pertussis antibodies. The etiologic agent of pertussis is the gram negative coccobacillus known as Bordetella pertussis. The bacteria is believed to invade the respiratory tract and induce a toxic state which remains even after the disappearance of the bacteria.

Although world health organizations presently recommend the immunization of infants to prevent the incidence and spread of pertussis, great concern has arisen over the negative effects of various available vaccine forms, i.e., the toxicity of conventional B. pertussis vaccine formulations causes side effects which vary from simple flushing to permanent neurological damage and/or death. Consequently, reduced use of conventional B. pertussis vaccines has resulted in a large increase in the number of the incidence of pertussis cases.

The current conventional pertussis vaccine contains whole but inactivated B. pertussis organisms. Such organisms are rendered nonviable after treatment at 56° C for 30 minutes and/or treatment with formaldehyde. However, since the bacteria are not subjected to any further detoxification treatment, any toxic substance which can withstand the elevated temperature is included in the vaccine. The presence of such toxic substances causes recipients of the the conventional vaccine to experience side effects which vary from simple flushing to permanent neurological damage and/or death.

Another available pertussis vaccine is prepared from the supernatant of B. pertussis culture media. However, variabilities in cultivation allow the final composition of the microorganisms to vary. Furthermore, the inactivating agents, gluteraldehyde or formaldehyde, occasionally lead to aggregated materials which are subject to conversion to active toxic substances.

Alternate available pertussis vaccines are prepared from avirulent or toxin deficient strains of B. pertussis. However, these vaccines have proven to be much less protective than those prepared from virulent strains. [See, e.g., Wardlaw et al., J. Med. Microbiol., 9:89-100 (1976)].

To avoid the side effects caused by whole cell vaccines, research turned to the investigation of the toxic components of the B. pertussis bacteria. One important component is pertussis toxin, a protein exotoxin which plays a major role in the pathogenesis of whooping cough and is believed to be the major protective antigen of B. pertussis. Pertussis toxin is a hexameric protein composed of five dissimilar subunits, called S1 through S5 according to their decreasing molecular weights. Pertussis toxin is structured in an A-B model, in which the B-oligomer (subunits S2 through S5) is responsible for the binding of the toxin to its receptor in the target cell membranes, and the A-protomer (subunit S1) contains enzymatic activity.

Studies with mice have shown that the administration of pertussis toxin alone provided protection against challenge with B. pertussis. [See. J.J. Munoz et al., Infect. Immun., 32:243-250 (1981)].

C. Locht et al., Nucleic Acids Research 14(A):3251-3261, (1986), discuss the cloning of a DNA fragment from the B. pertussis toxin gene containing at least subunit S4 and a portion of another subunit gene.

Nicosia et al., Proc. Natl. Acad. Sci. USA, 83:4631-4635 (1986), discuss the cloning and sequencing of a 4.7 kb DNA fragment containing genes coding for the five pertussis toxin subunits.

C. Locht et al., Science 232:1258-1264 (1986), determined the DNA sequence and deduced the protein sequence of the pertussis toxin gene.

C. Locht et al., Infect. Immun. 55:2546-2553 (1987), discuss the expression of individual pertussis toxin subunit genes in E. coli using plasmids encoding subunits S1 and S2 as proteins with minimal fusions to the amino terminal residues of beta-galactosidase. The expressed S1 polypeptides from these plasmids all contained the six amino terminal amino acids from beta-galactosidase followed by five amino acids encoded by a polylinker.

Efforts to detoxify pertussis toxin and exploit its immunoprotective potential have been attempted by treatment with formaldehyde, thimerosal [See, WHO Expert Committee on Biological Standardization, "Requirements for pertussis vaccine", Tech. Rep. Ser., 638:60 (1979)], or glutaraldehyde [See, Relyveld, E.H. and Ben-Efraim, S., Meth. Enzymol. 93:24 (1983)]. However, efficiency of such treatment is not always reproducible and reversions are common.

United States Patent Application S.N. 874,637, titled "Novel Method of Preparing Toxoid", discloses a B. pertussis vaccine containing pertussis toxin inactivated by an oxidizing agent such as hydrogen peroxide. The resulting composition is a chemically irreversible antigen which is non-toxic, stable, immunogenic and protective against pertussis infection.

Keith et al., United States Patent Application Serial Number 843,727, filed March 25, 1986, describe a pertussis toxin gene or portions thereof useful for cloning and expression in the production of pure antigen peptide which co-migrates as a major band with subunit S4 of the pertussis toxin, for use in a vaccine.

C. Locht et al., Infection and Immunity, 55 (11):2546-2553 (Nov. 1987), discloses the expression of the S1 and S2 subunits of pertussis toxin in E. coli. The recombinant S1 subunit contained the enzymatic NAD glycohydrolase and ADP-ribosyltransferase activities. A truncated version of the S1 subunit is described in which the 48 carboxy terminal amino acid residues were deleted.

W.J. Black et al., Infection and Immunity, 55(10):2465-2470 (Oct. 1987), introduced mutations in the cloned pertussis toxin gene by allelic exchange into the chromosome of B. pertussis. A series of B. pertussis strains were isolated which were isogenic. One of the plasmids which was transformed into E. coli contained all the genes for subunits S2, S3, S4 and S5 and only a portion of the gene for subunit S1. A truncated S1 subunit was formed in the absence of an S5 subunit in another mutation of the toxin gene.

Sclavo SpA, European Patent Application Publication Number 232,229, published August 12, 1987, discloses the cloning and expression of B. pertussis toxin encoding DNA having 4696 base pairs and containing the S1 through S5 subunits of the toxin.

J.T. Barbieri et al., Infection and Immunity, 55(5):1321-1323 (May 1987), disclose the expression of the S1 subunit of the pertussis toxin as a fusion protein in a strain of E. coli deficient in protein degradation. This protein contains the first six amino acids of beta-galactosidase and 5 amino acids from the pUC18 polylinker followed by amino acids 2-235 of the S1 subunit. Expression of the fusion protein is controlled by the beta-galactosidase promoter.

A. Nicosia et al., Infection and Immunity, 55(4):963-967 (April 1987), disclose expression of the five subunits of B. pertussis as fusion proteins in E. coli and the production of polyvalent sera against them.

Institut Pasteur, PCT Patent Application Publication Number WO8703301, published June 4, 1987, disclose recombinant phages containing B. pertussis DNA for production of whooping cough vaccines. The expression products are fusion proteins with the characteristics of adenylatecyclase, leucocytosis promoting factor, and FHA. The products are encapsidated in lambda phage capsids to obtain recombinant phages.

J. Riser et al., in Vaccines 86: New Approaches to Immunization, F. Brown et al., Eds. Cold Spring Harbour Laboratory, pages 235-238 (1986), describe the initial characterization of an E. coli clone after cloning of an antigenic determinant specific for the pertussis toxin polypeptide S2.

Burnette et al., Biotechnology, 6, 699-708 (1988), discuss the subcloning and direct expression of the individual subunits of B. pertussis in E. coli.

There remains a continuing need for effective and safe vaccines against infection caused by B. pertussis.

SUMMARY OF THE INVENTION

The present invention relates to a B. pertussis toxin coding sequence containing an entire or truncated B. pertussis toxin S1 subunit amino acid coding sequence provided that (a) the S1 subunit coding sequence contains a modification of the tryptophan residue at amino acid position 26 (hereinafter "Trp-26"), and (b) the Trp-26 modification results in substantially inactivated S1 enzymatic activity in the protein encoded by the B. pertussis toxin coding sequence but such protein has retained the capacity to be recognized by anti-pertussis toxin antibodies. This invention also relates to a recombinant DNA molecule containing the coding sequence of this invention.

As a further embodiment of the invention, there is provided a recombinant DNA plasmid comprising the coding sequence of this invention. This plasmid preferably contains the above coding sequence in operative association with suitable expression control sequences. By 'suitable expression control sequences' is meant those sequences which are required to enable expression of the coding sequence of this invention by a suitable host cell into which the plasmid of this invention is introduced. Such expression control sequences are well known in the art.

Another aspect of the invention is a method of producing a transformed host cell which comprises transforming a desired host cell with the plasmid of the invention.

Still a further aspect of this invention is a host cell transformed with the plasmid of this invention. Such host cell is capable of growth in a suitable culture medium and expressing the coding sequence of this invention.

Yet a further aspect of the invention involves a method for producing the protein of the invention which comprises culturing the transformed host of the invention in a suitable culture medium and isolating the protein so produced. The present invention also relates to the protein encoded by the coding sequence of this invention.

Another embodiment of the invention is a vaccine for stimulating protection against whooping cough, wherein such vaccine comprises an immunoprotective and non-toxic amount of the protein of the invention. Such vaccine may comprise such protein alone or in conjunction with other antigens and/or adjuvants. By the term immunoprotective amount' is meant an amount of any protein of this invention which will elicit a sufficient protective immune response against pertussis after such amount of such protein is administered to a human host.

In addition, the present invention also provides a method for inoculating a human against whooping cough which comprises administering the vaccine of this invention to such human.

Another embodiment of this invention is a process for producing the protein encoded by a Bordetella pertussis toxin amino acid coding sequence containing an entire or truncated B. pertussis toxin S1 subunit amino acid coding sequence provided that (a) the S1 subunit coding sequence contains a modification of the tryptophan residue at amino acid position 26 (hereinafter "Trp-26"), and (b) the Trp-26 modification results in substantially inactivated S1 enzymatic activity in the protein encoded by the B. pertussis toxin operon amino acid coding sequence but such protein has retained the capacity to be recognized by anti-pertussis toxin antibodies, wherein such process comprises (i) treating a Bordetella pertussis toxin amino acid coding sequence, prepared by isolation from native B. pertussis DNA or by synthetic or recombinant DNA techniques, containing an entire or truncated B. pertussis toxin S1 subunit amino acid coding sequence provided that the S1 subunit coding sequence does not already contain a modification of the tryptophan residue at amino acid position 26, with site-directed mutagenesis, a tryptophan modifying chemical reagent or photooxidation to produce a modification or deletion of the tryptophan residue at amino acid position 26, and transforming a desired host cell with a plasmid containing such resulting coding sequence and culturing such host in suitable culture medium and isolating the protein so produced, (ii) substituting or deleting the tryptophan residue at position 26 of the S1 subunit of a Bordetella pertussis toxin amino acid coding sequence, prepared by isolation from native B. pertussis DNA or by synthetic or recombinant DNA techniques, containing an entire or truncated B. pertussis toxin S1 subunit amino acid coding sequence with another amino acid, and transforming a desired host cell with a plasmid containing such resulting coding sequence and culturing such host in suitable culture medium and isolating the protein so produced, or (iii) preparing such coding sequence by synthetic methods and transforming a desired host cell with a plasmid containing such coding sequence and culturing such host in suitable culture medium and isolating the protein so produced. This invention also relates to the protein produced by such process, a vaccine for stimulating protection against whooping cough wherein such vaccine comprises an immunoprotective and non- toxic quantity of such protein, and a method for innoculating a human against subsequent whooping cough which comprises administering the vaccine to such human.

A further aspect of this invention is a B. pertussis strain in which the chromosomal pertussis toxin gene is inactivated, wherein such strain contains the recombinant DNA molecule of this invention. Another embodiment of the invention is a whole cell vaccine for stimulating protection against whooping cough, wherein such vaccine comprises an immunoprotective and non-toxic amount of the strain of the invention. Such vaccine may comprise such strain alone or in conjunction with other antigens and/or adjuvants. Still another aspect of this invention relates to a method for innoculating a human against subsequent whooping cough which comprises administering the vaccine to such human.

Other aspects and advantages of the present invention will be disclosed in the following detailed description of the presently preferred embodiment thereof.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the the discovery that modification of the naturally occurring tryptophan amino acid residue at position 26 of the S1 subunit of the pertussis toxin coding sequence greatly reduces the toxicity of pertussis toxin while retaining its ability to elicit a protective immunogenic response.

Pertussis toxin is a protein exotoxin of B. pertussis which plays a major role in the pathogenesis of whooping cough and is believed to be the major protective antigen of B. pertussis. [See, A.A. Weiss et al., Ann. Rev. Microbiol., 40:661 (1986)]. Pertussis toxin is a hexameric protein composed of five dissimilar subunits called S1 through S5 according to their decrea sing molecular weights. [See. M. Tamura et al., Biochem., 21:5516 (1982)]. Pertussis toxin is structured in an A-B model in which the B-oligomer (subunits S2 through S5) is responsible for the binding of the toxin to its receptor in the target cell membranes, and the A-protomer (S1 subunit) contains enzymatic activity.

It has now been found that due to the Trp-26 modification of the S1 subunit contained by the coding sequence of this invention, the coding sequence encodes a protein which has substantially inactivated S1 subunit enzymatic activity. The normal S1 subunit enzymatic activity contributes to the toxicity of a variety of B. pertussis vaccines containing pertussis toxin or S1 subunit protein. Such enzymatic activity includes NAD-glycohydrolysis or ADP-ribosyltransfer. Thus, the vaccine of this invention has no such toxicity problems. Additionally, such inactivation of the S1 subunit enzymatic activity nevertheless allows the protein derived from the coding sequence of this invention to retain recognition by anti-pertussis toxin antibodies.

The present invention thus describes a B. pertussis toxin amino acid coding sequence containing an entire or truncated B. pertussis toxin S1 subunit amino acid coding sequence provided that (a) the S1 subunit coding sequence contains a modification of the tryptophan residue at amino acid position 26 (hereinafter "Trp-26"), and (b) the Trp-26 modification results in substantially inactivated S1 subunit enzymatic activity in the protein encoded by the B. pertussis toxin coding sequence but such protein retains the capacity to be recognized by anti-pertussis toxin antibodies. This invention also describes a recombinant DNA molecule comprising the coding sequence of this invention in addition to the B. pertussis toxin amino acid coding sequence of this invention, the recombinant DNA mole cule of the invention may comprise additional DNA sequences, including, e.g., a regulatory element, one or more selection markers, and replication and maintainance functions.

By the term "B. pertussis toxin coding sequence" as used in the description of the coding sequence of this invention is meant the entire B. pertussis toxin operon or any functional truncated fragment thereof which contains an entire or truncated B. pertussis toxin S1 subunit amino acid coding sequence provided that (a) the S1 subunit coding sequence contains a modification at Trp-26, and (b) the Trp-26 modification results in substantially inactivated S1 subunit enzymatic activity in the protein encoded by the B. pertussis toxin coding sequence but such protein retains the capacity to be recognized by anti-pertussis toxin antibodies. By the term "B. pertussis toxin coding sequence" as used in the description of the coding sequence of this invention is also meant any functional fusion protein coding sequence comprising (i) the entire B. pertussis toxin coding sequence or any functional truncated fragment thereof which contains an entire or truncated B. pertussis toxin S1 subunit coding sequence, and (ii) any other protein coding sequence, provided that (a) the S1 subunit coding sequence contains a modification at Trp-26, and (b) the Trp-26 modification results in substantially inactivated S1 subunit enzymatic activity in the protein encoded by the B. pertussis toxin coding sequence but such protein retains the capacity to be recognized by anti-pertussis toxin antibodies. By the term "functional" is meant any B. pertussis toxin coding sequence which encodes a protein which will either be sufficiently recognizable by anti-pertussis toxin antibodies to be useful in a diagnostic assay for B. pertussis, and/or will elicit a sufficient protective immune response against pertussis after an immunoprotective amount of such protein is administered to a human host.

Preferably, the B. pertussis toxin coding sequence of this invention is a truncated version of the B. pertussis toxin operon comprising only the B. pertussis toxin S1 subunit amino acid coding sequence or a truncated portion thereof. Other preferred embodiments of the B. pertussis toxin amino acid coding sequence of this invention include the complete B. pertussis operon, or the coding sequence of any subunit thereof either alone or in conjunction with one or several other subunits. Another preferred embodiment of the coding sequence of this invention is one in which also contains a modification of the glutamic acid residue at amino acid position 129 of the S1 subunit coding sequence.

The nucleotide coding sequence of the entire pertussis toxin operon has been determined. See. e.g., Keith, et al., United States Patent Application Serial No. (U.S.S.N.) 843,727, filed March 26, 1986, the entire disclosure of which is hereby incorporated by reference. Furthermore, DNA containing the pertussis toxin coding sequence may be isolated from any publicly available B. pertussis strain. For example. various strains of B. pertussis are publicly available from commercial depositories, e.g., from the American Type Culture Collection, Rockville, Maryland, U.S.A. Such isolation may be effected by the method of Keith, et al., U.S.S.N. 843,727, supra, or any other conventional method.

Modification of the Trp-26 residue of the S1 subunit portion of the coding sequence of this invention may be effected in a variety of ways utilizing conventional techniques.

For example, one method of alteration of the Trp-26 residue is by use of a tryptophan (Trp) modifying chemical reagent such as 2-hydroxy-5-nitrobenzyl bromide (HNBB). In one embodiment of the invention, the catalytic domain for the NAD-glycohydrolase and ADP-ribosyltransferase activities of the pertussis toxin S1 subunit, e.g., amino acid residues 2-187 of the S1 subunit, was purified from an E. coli strain that expresses the gene coding for this catalytic domain. This purified polypeptide was treated with a tryptophan modifying chemical reagent (HNBB) to produce a modification of the Trp-26 residue. When the polypeptide resulting from the HNBB treatment was subsequently assayed for its S1 enzymatic activities, it was determined that it had no such activities, i.e., both NAD-glycohydrolase and ADP-ribosyltransferase activities were abolished as measured by standard assay procedures.

Additional tryptophan modifying chemical reagents which can be employed to effect modification of the Trp-26 residue include, but are not limited to, N-Bromosuccinimide (See, e.g., Spande, T.F. & Withop, B., 1967, Meth. Enzymol., 11:522), H_2O_2, performic acid, sulfenyl halides (See, e.g., Seolfone, E., Fontana, A. & Roccki, R., 1968, Biochemistry, 7:971), 2-nitrophenylsulfenyl chloride, 2,4-dinitrophenylsulfenyl chloride, and 2-acetoxy-5 nitrobenzyl chloride.

Photooxidation may also be employed as a method for modifying the Trp-26 residue of the S1 subunit. (See, e.g., Spikes, J.D. & Straight, R., 1967, Ann. Rev. Phys, Chem. 18:409).

Yet another means of modifying the Trp-26 residue of the S1 subunit includes the deletion or substitution of the codon for that residue in the gene coding for S1 subunit by conventional site-directed mutagenesis. One example of the performance of site directed mutagenesis on an exemplary construct of the coding sequence of this invention in the form of an S1 subunit gene is described in Example 5. For example, the S1 subunit coding sequence was subcloned into an M13 vector so that expression of this sequence could be produced in E. coli transformed by the recombinant M13 vector. The Trp-26 residue was then deleted, or changed to threonine (Thr), by site directed mutagenesis. Other vector constructs of the invention may be conventionally designed by one of skill in the art to perform such site-directed mutagenesis at the Trp-26 site.

An alternative method for modification of the tryptophan residue at position 26 of the S1 subunit coding sequence to create the coding sequence of the invention includes the deletion of the Trp-26 residue and its substitution by threonine (Thr) by conventional genetic engineering techniques or substitution at that site by other suitable amino acid residues. Other suitable substitute amino acids include, but are not limited to, glutamine (Gln), tyrosine (Tyr) and phenylalanine (phe). A Trp-26 to Gln change is a more conservative replacement in terms of secondary structure propensity. A Trp to Tyr change may maintain an intermediate polarity characteristic, while a Trp to Phe change maintains the aromatic ring. The modified molecule should resemble the natural molecule as much as possible, therefore one would like a very similar secondary

structure, hydrophobicity profile and proportion of aromatic amino acids. Any other amino acid substitutions may be considered provided that the substitution results in the creation of a coding sequence encoding a protein with substantially inactivated S1 enzymatic activity and retention of the anti-B. pertussis toxin antibody recognition.

The amino acid coding sequence of this invention may also be produced (a) synthetically, using conventional DNA synthesis techniques, or (b) by other chemical modification methods using conventional chemical modification techniques.

Additionally, by employing conventional genetic engineering techniques, the coding sequence of this invention may be in the form of any truncated version of the pertussis toxin operon containing the Trp-26 modification, or in the form of a truncated version of the S1 subunit coding sequence containing only the codons for amino acids 2 through 187 of the S1 subunit coding sequence including the Trp-26 modification, or alternatively, the coding sequence of this invention may contain any truncated portion of the S1 subunit coding sequence including the Trp-26 modification.

Furthermore, the coding sequence of this invention may be in the form of a double mutant, i.e., a coding sequence containing both a Trp-26 modification as well as a modification of another amino acid residue in the S1 subunit provided that the protein encoded by such coding sequence retains the capacity to be recognized by anti-pertussis toxin antibodies. Preferably, the protein encoded by such coding sequence also exhibits a decrease in S1 subunit enzymatic activity. Examples of such additional modifications which may be employed include, but are not limited to, modification of the glutamic acid residue at amino acid position 129 of the S1 subunit coding sequence (Glu-129); modification of the histidine residue at amino acid position 35 of the S1 subunit coding sequence (His-35); modification of the arginine residue at amino acid position 9 of the S1 subunit coding sequence (Arg-9) and modification of the serine residue at amino acid position 40 of the S1 subunit coding sequence (Ser-40). Modification of the glutamic acid residue may be effected by conventional techniques such as those referred to supra. See, also, Example 13, infra, and Pizza et al., Proc. Natl. Acad. Sci. USA:85, 7521-7525 (1988).

Moreover, the coding sequence of the invention may appear in the form of a fusion protein coding sequence comprising the B. pertussis toxin amino acid coding sequence fused to at least one other coding sequence, such as, but not limited to, a pertussis toxin derived coding sequence and/or a non-pertussis toxin derived coding sequence. For example, such fusion protein may comprise all or part of the S1 subunit coding sequence including the Trp-26 modification fused to all or a portion of the S2 subunit coding sequence and/or the S3 subunit coding sequence and/or the S4 subunit coding sequence and/or the S5 subunit coding sequence. Such fusion protein may also comprise all or part of the S1 subunit coding sequence including the Trp-26 modification fused to other antigens of B. pertussis or other antigens such as, but not limited to, Diphtheria antigens and/or Tetanus antigens and/or Haemophilus influenzae antigens. An example of a fusion protein coding sequence of this invention is the one described in the examples which contains the following sequence:

Sequence:

```
ATG   ACC ATG ATT ACG AAT TCG AGC TCG GTA CCC GGG GAT
(Met) Thr Met Ile Thr Asn Ser Ser Ser Val Pro Gly Asp
          beta-galactosidase    polylinker       ____2
                                                     S1

CCT CCC GCC ACC GTA...... GTA GCG TCG ATC CTC TAG
Pro Pro Ala Thr Val...... Val Ala Ser Ile Leu Stop
 3   4   5   6   7        184 185 186 187 polylinker
        S1
```

Other coding sequences of this invention which are in the form of a fusion protein can be conventionally designed and prepared by one of skill in the art.

Furthermore, the above Trp-26 modified S1 truncated fragment can be associated with other sequences of the wild-type pertussis toxin operon such as any other of the S2-S5 subunit coding sequences alone or in conjunction with one or more of the S2 through S5 subunits coding sequences. Alternatively, a complete B. pertussis toxin coding sequence may be generated containing (a) the above Trp-26 modified S1 truncated fragment replacing the naturally occurring S1 cistron or, (b) a complete S1 subunit cistron containing only the Trp 26 modification or some other fragment containing such a Trp-26 modification in association with other

components.

Still a further aspect of this invention is a host cell transformed with the plasmid of this invention. Such host cell is capable of growth in a suitable culture medium and expressing the coding sequence of the invention. Such host cell is prepared by the method of this invention, i.e., transforming a desired host cell with the plasmid of the invention. Such transformation is accomplished by utilizing conventional transformation techniques. The most preferred host cells include those belonging to the species E. coli. and the genus Bordetella. Other host cells which may be suitable include, but are not limited to, mammalian cells, insect cells, other bacterial cells and yeast cells. Thus, this invention is not limited to any specific host cells.

The present invention also relates to a method of producing the protein encoded by the coding sequence of this invention which comprises culturing the transformed host of the invention in appropriate culture media and isolating such protein. By "appropriate culture media" is meant that media which will enabled the transformed host to grow and which will also enable such host to express the coding sequence of the invention in recoverable quantity. It will be appreciated by one of skill in the art that the appropriate culture media to use will depend upon the host cell employed. The isolation of the protein so produced is accomplished from a culture lysate of the host or, if appropriate, directly from the host's culture medium, and is carried out by conventional protein isolation techniques.

Thus, in view of the above description, another embodiment of this invention is a process for producing the protein encoded by a Bordetella pertussis toxin amino acid coding sequence containing an entire or truncated B. pertussis toxin S1 subunit amino acid coding sequence provided that (a) the S1 subunit coding sequence contains a modification of the tryptophan residue at amino acid position 26 (hereinafter "Trp-26"), and (b) the Trp-26 modification results in substantially inactivated S1 enzymatic activity in the protein encoded by the B. pertussis toxin operon amino acid coding sequence but such protein has retained the capacity to be recognized by anti-pertussis toxin antibodies, wherein such process comprises (i) treating a Bordetella pertussis toxin amino acid coding sequence, prepared by isolation from native B. pertussis DNA or by synthetic or recombinant DNA techniques, containing an entire or truncated B. pertussis toxin S1 subunit amino acid coding sequence, provided that the S1 subunit coding sequence does not already contain a modification of the tryptophan residue at amino acid position 26, with site-directed mutagenesis, a tryptophan modifying chemical reagent or photooxidation to produce a modification or deletion of the tryptophan residue at amino acid position 26, and transforming a desired host cell with a plasmid containing such resulting coding sequence and culturing such host in suitable culture medium and isolating the protein so produced, (ii) substituting or deleting the tryptophan residue at position 26 of the S1 subunit of a Bordetella pertussis toxin amino acid coding sequence, prepared by isolation from native B. pertussis DNA or by synthetic or recombinant DNA techniques, containing an entire or truncated B. pertussis toxin S1 subunit amino acid coding sequence with another amino acid, and transforming a desired host cell with a plasmid containing such resulting coding sequence and culturing such host in suitable culture medium and isolating the protein so produced, or (iii) preparing such coding sequence by synthetic methods and transforming a desired host cell with a plasmid containing such resulting coding sequence and culturing such host in suitable culture medium and isolating the protein so produced. This invention also relates to the protein produced by such process, a vaccine for stimulating protection against whooping cough wherein such vaccine comprises an immunoprotective and non-toxic quantity of such protein, and a method for innoculating a human against subsequent whooping cough which comprises administering the vaccine.

The invention also encompasses a vaccine capable of inducing immunity against pertussis. Such vaccine comprises an immunoprotective and non-toxic amount of the protein of the invention regardless of the method of preparation of such protein. Such vaccine preferably contains 5-25 µg of the protein of this invention, but is not limited to these amounts.

Other antigens which are known to be desirably administered in conjunction with pertussis toxin may also be included in the vaccines. Such additional components are known to those of skill in the art. Preferable additional components include tetanus toxoid and/or diphtheria toxoid as well as filamentous haemagglutinin (FHA) and/or any other protective antigen of B. pertussis.

The provision of such a vaccine thus allows another aspect of the present invention, i.e., a method for immunizing a human against pertussis which comprises administering the vaccine of the subject invention to such human.

The mode of administration of the vaccine of the invention may be any suitable route which delivers an immunoprotective amount of the protein of the subject invention to the host. However, the vaccine is preferably administered parenterally via the intramuscular or deep subcutaneous routes. Other modes of administration may also be employed, where desired, such as oral administration or via other parenteral routes, i.e., intradermally, intranasally, or intravenously.

The vaccine of the invention may be prepared as a pharmaceutical composition containing an immunoprotective, non-toxic amount of the protein of the invention in a nontoxic and sterile pharmaceutically acceptable carrier. Where the administration of the vaccine is parenteral, the protein of the invention can be optionally admixed or absorbed with any conventional adjuvant, for use as a non-specific irritant to attract or enhance an immune response. Such adjuvants include, among others, aluminum hydroxide, aluminum phosphate, muramyl dipeptide and saponins, such as Quil A. As a further exemplary alternative of the preparation of the vaccine of the invention, the protein of the invention can be encapsulat d within microparticles, such as liposomes. In yet another exemplary alternative of the preparation of the vaccine of the

invention, the protein of the invention can be administered with an immunostimulating macromolecule, or a tetanus toxoid. Alternatively, an aqueous suspension or solution containing the protein of the invention preferably buffered at physiological pH, may be designed for oral ingestion.

It is preferred that the vaccine of the invention, when in a pharmaceutical preparation, be present in unit dosage forms. The appropriate therapeutically effective dose can be determined readily by those of skill in the art. The effective amount of protein contained in the vaccine of this invention may be in the range of the effective amounts of antigen in conventional B. pertussis acellular or component vaccines, i.e., 5-25 µg of protein. This dose may optionally be delivered with various amounts of filamentous haemagglutinin (FHA) (about 10 ug-25 ug) and/or agglutinogens or other antigens. It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the age, general health, sex, and diet of the patient; the time of administration; the route of administration; synergistic effects with any other drugs being administered; and the degree of protection being sought. Of course, the administration can be repeated at suitable intervals if necessary. Preferably, three doses will be administered, with 3 to 12 months intervals between each dose, with an optional booster dose later in time.

A further aspect of this invention is a B. pertussis strain in which the chromosomal pertussis toxin gene is inactivated, wherein such strain contains the recombinant DNA molecule of this invention. Another embodiment of the invention is a whole cell vaccine for stimulating protection against whooping cough, wherein such vaccine comprises an immunoprotective and non-toxic amount of the strain of the invention. Such vaccine may comprise such strain alone or in conjunction with other antigens and/or adjuvants. Still another aspect of this invention relates to a method for innoculating a human against subsequent whooping cough which comprises administering the vaccine to such human. Any strain of B. pertussis which contains the chromosomal pertussis gene may be employed as the starting material for preparing the strain of the subject invention. Numerous suitable strains of B. pertussis are publicly available from various repositories such as the American Type Culture Collection, Rockville, Maryland, U.S.A. The preparation of the strain my be carried out by conventional techniques. For example. to prepare the strain of the subject invention, the chromosomal pertussis toxin gene is inactivated by conventional techniques, such as by deletion, point or insertion mutation, and the recombinant DNA molecule of this invention is transformed into such strain by conventional techniques. It is preferable to use a B. pertussis strain in which the chromosomal toxin gene is deleted, rather than inactivated by point or insertion mutations. Inactivation of the gene through point or insertion mutations does not eliminate DNA sequences that have the potential of recombining in vivo with the DNA coding for the mutated pertussis toxin on a autonomously replicating plasmid. On the other hand, in a pertussis toxin gene deletion mutant, no such recombi nation can occur. The resulting B. pertussis strain is then transformed with the recombinant DNA molecule of this invention to result in the strain of this invention. It is preferable if such recombinant DNA molecule is comprised by the plasmid of this invention. Such recombinant DNA molecule may be integrated into the chromosome of the host cell or, if in the form of a plasmid, may be maintained episomally. After such recombinant DNA molecule are introduced, the resulting B. pertussis strain may be used to produce the mutated pertussis toxin protein of this invention. Alternatively, after such recombinant DNA molecule is introduced, such strain may now be used to prepare a whole cell vaccine against pertussis. Preparation of such a whole cell vaccine is accomplished by conventional techniques.

The provision of such a vaccine thus allows another aspect of the present invention, i.e., a method for immunizing a human against pertussis which comprises administering the vaccine of the subject invention to such human.

The mode of administration of the vaccine of the invention may be any suitable route which delivers an immunoprotective amount of the strain of the subject invention to the host. However, the vaccine is preferably administered parenterally via the intramuscular or deep subcutaneous routes. Other modes of administration may also be employed, where desired, such as oral administration or via other parenteral routes, i.e., intradermally, intranasally or intravenously.

The appropriate immunoprotective and non-toxic dose of such vaccine can be determined readily by those of skill in the art i.e., the appropriate immunoprotective and non-toxic amount of the strain of this invention contained in the vaccine of this invention may be in the range of the effective amounts of antigen in conventional B. pertussis whole cell vaccines. This dose may optionally be delivered with various amounts of filamentous haemagglutinin (FHA) (about 10 ug-25 ug) and/or agglutinogens or other antigens. It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the age, general health, sex, and diet of the patient; the time of administration; the route of administration; synergistic effects with any other drugs being administered; and the degree of protection being sought. Of course, the administration can be repeated at suitable intervals if necessary. Preferably, three doses will be administered, with 3 to 12 months intervals between each dose, with an optional booster dose later in time.

EXAMPLES

The following examples illustrate the preparation of the coding sequence, vector, transformed host and protein of the invention; antigenic activity of the protein; and preparation of the vaccine of the invention. These

examples are illustrative in nature and are not intended to limit the scope of this invention.

In the following Examples, all temperatures are in degrees Centigrade (Celsius). The enzymes used in DNA manipulations were obtained from Boehringer (Mannheim, West Germany) and Amersham (Amersham, England), and were employed according to the supplier's directions.

In the following examples, the following abbreviations may be employed:

YT medium: 8 grams/liter (g/l) Tryptone, 5 g/l NaCl, 5g/l yeast extract

Tris-HCl 1 M stock solution : 121,1 g/l Trizma base + HCl until pH reaches desired value (example 7.5).

LB medium (pH 7.5): Tryptone 10 g/l; yeast extract 5 g/l; NaCl 10 g/l

Example 1. Construction of expression vector pRIT20001 encoding S1 subunit-related protein

Plasmid pPTX42, described in Locht, C. et al., Nucleic Acids Res., 14:3251 (1986), was digested into fragments with Sau3a. The fragments were resolved by polyacrylamide gel electrophoresis (PAGE) according to the method of Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor, NY, (1982). A 560 base pair (bp) fragment was purified and inserted into the replicative form of M13mp18, previously digested with BamHI. M13mp18 is described in Messing, J. Methods Enzymol., 101, 20-78, (1983) and was obtained from Bethesda Research Laboratories, Bethesda, Maryland. E. coli TG1 cells, obtained from Amersham, International PLC, Amersham, United Kingdom, were transformed with the replicative form of the recombinant phages according to the technique of Maniatis et al., supra. After transformation and overnight incubation, the phages were analyzed for the presence of the 560 bp insert by DNA sequencing according to the method of Sanger et al., Proc. Natl. Acad. Sci., U.S.A., 74, 5463-5467, (1977).

One phage containing the 560 bp insert was purified and was named pRIT20001. It was determined that the S1 subunit-related protein encoded by pRIT20001 was identical to rS1d, the S1 subunit-related protein encoded by pTXS11 as described in Locht et al., Infect. Immun., 55;2546 (1987), the entire disclosure of which is hereby incorporated herein by reference. In this construct, i.e., pRIT20001, the six amino-terminal amino acids are the first six amino acids from beta-galactosidase. They are followed by five amino acids encoded by the polylinker sequence in the M13mp18 cloning vector, after which residues 2 to 187 of the pertussis toxin S1 subunit coding sequence are present. Finally a Leu residue is encoded by the polylinker and a Stop codon is provided. This gives the following sequence for the S1 subunit-related protein:

Example 2. Synthesis of S1 related protein in E. coli using pRIT20001

1.5 ml of an overnight culture of E. coli TG1 cells were inoculated in 100 ml YT medium, and were thereafter infected with 10 µl of pRIT20001, prepared as described in Example 1, or M13mp18 phage stock. The infected cells were grown under constant shaking at 37° until the Optical Density (OD) reached 0.3. Isopropyl-beta-D-galactopyranoside (IPTG) was then added to the cells to a final concentration of 1 mM, and growth was continued for 4 to 5 hours. The cells were then harvested by centrifugation, resuspended in 3 ml lysis buffer (25 mM Tris-HCl (pH 7.5), 25 mM NaCl) and lysed by two passages through a French pressure cell. The lysate was fractionated by centrifugation at 12,000 x G for 40 minutes.

The supernatant fraction was then analyzed for the presence of the S1 related protein by Western blot according to the method of Towbin et al., Proc. Natl. Acad-Sci., U.S.A., 76:4350-4354, (1979), and detected with an anti-S1 monoclonal antibody B2F8 [See. Marchitto, K.S. et al., Infect. Immun. 55:1309 (1987)]. provided by Dr. J.M. Keith, Laboratory of Pathobiology, NIAID, Rocky Mountain Laboratories, Hamilton, Montana. Supernatant fractions from cells infected with M13mp18 did not contain a protein specifically recognized by monoclonal antibody B2F8. However, supernatant fractions from cells infected with pRIT20001 contained a protein of about 22,000 daltons that reacted with mono clonal antibody B2F8 on the Western blot. This protein behaved the same way with monoclonal antibody B2F8 on the Western blot as the S1 subunit-related protein, rS1d, encoded by pTXS11 [see, Locht, C. et al., Infect. Immun., (1987), supra.]

Example 3. Enzymatic activities of S1 subunit-related protein synthesized in E. coli infected by pRIT20001

The supernatant fraction described in Example 2 were assayed for the presence of S1 subunit-related protein specific enzymatic activities. The level of these activities were compared to the activities of rsld which had been partially purified from E. coli cells containing pTXS11. In the nicotinamide adenine dinucleotide (NAD)-glycohydrolase assay described in Locht et al., Infect. Immun., (1987), supra, the release of nicotinamide from NAD catalyzed by such partially purified rsld was measured. 1 µg of such partially purified rsld catalyzed the release of 1,200 pmoles of nicotinamide after three hours of reaction at 30° in the assay conditions described in Locht et al., Infect. Immun., (1987), supra. The supernatant fraction of TG1 cells infected with M13mp18 contained no significant NAD-glycohydrolase activity, and the measured nicotinamide release values were not different from the background levels. 25 µl of the supernatant fraction of E. coli TG1 cells infected with pRIT20001, however, catalyzed the release of 300 pmoles nicotinamide from NAD after three hours of incubation in the same conditions. The substantial reduction of the enzymatic activity by the supernatant of pRIT20001 infected E. coli cells is due to the presence of an inhibitor in the crude E. coli cell extracts (C. Locht, unpublished observation).

The ability of the partially purified rsld and the supernatant fractions of pRIT20001 infected E. coli cells and M13mp18 infected TG1 cells to catalyze the S1-specific adenosine diphosphate (ADP)-ribosylation of the 41,000 dalton signal transducing GTP-binding protein Gi in CHO cell membranes was also investigated. This assay is also described in Locht et al., Infect. Immun., (1987), supra. When the partially purified rSd or pertussis

toxin was incubated in the presence of radiolabeled NAD and Chinese Hamster Ovary (CHO) cell membranes in the conditions described in Locht et al., and the products of the reaction were analyzed by sodium dodecyl sulfate-PAGE (SDS-PAGE) and autoradiography, specific labeling of the 41,000 dalton Gi protein was detected. No labeling of the Gi protein was seen when rS1d or pertussis toxin was replaced by the lysis buffer (25 mM Tris-HCl (pH7.5), 25 mM NaCl) or the supernatant fraction of E. coli cells infected with M13mp18. In contrast, the supernatant fraction of cells infected with pRIT20001 (containing the protein encoded by a truncated S1 subunit coding sequence) was able to catalyze the incorporation of radiolabeled ADP-ribose in the 41,000 dalton Gi protein.

Example 4. Chemical modification of residue Trp-26 in the S1 subunit-related protein encoded by pRIT20001

The enzymatically active S1 related protein encoded by pRIT20001, and prepared according to Example 2, contains only a single Trp residue located at position 26 of the mature S1 protein, i.e., 15 residues upstream from the cysteine residue at position 41.

The S1 subunit-related protein prepared according to Example 2 was partially purified and incubated with a 1000-fold molar excess of 2-hydroxy-5-nitrobenzyl bromide (HNBB) dissolved in dry acetone. HNBB modifies tryptophan residues by derivatization. The reaction was performed in a 0.1 M sodium acetate buffer (pH 4.0), containing 2M urea at 25° for 2 hours. The solution was then dialyzed against 0.1 M sodium phosphate (pH 4.0) for 15 hours at 4°. In parallel, the S1 subunit-related protein prepared according to Example 2 was treated the same way, except that HNBB was omitted in the reaction mixture.

The S1 subunit-related protein prepared according to Example 2 as well as purified HNBB treated and HNBB untreated S1 subunit-related protein prepared as described above, hereinafter referred to as "untreated S1 subunit related protein" "HNBB treated S1 subunit-related protein" and "HNBB untreated S1 subunit-related protein", respectively, were then analyzed for their ability to catalyze NAD-glycohydrolysis and ADP-ribosyl-transfer to Gi in CHO cell membranes using assay conditions as described in Locht et al., Infect. Immun., (1987), supra. Untreated S1 subunit-related protein as well as HNBB untreated S1 subunit-related protein expressed NAD-glycohydrolase activity in a concentration-related fashion. Only marginal NAD-glycohydrolase activity could be detected when HNBB treated S1 subunit-related protein was assayed, and the activity was reduced to about 5% when a smaller concentration, i.e., 1 µg, of the HNBB treated S1 subunit-related protein was assayed. When 0.5 µg and 1.0 µg of untreated, HNBB treated and HNBB untreated S1 subunit-related protein were assayed for their ADP-ribosyltransferase activity on the Gi protein in CHO cell membranes, only the untreated S1 subunit-related protein and the HNBB untreated S1 subunit related protein were found to express Gi-specific ADP-ribosylation activity at both concentrations. No Gi : ADP-ribosyltransferase activity could be detected at either concentration of HNBB treated S1 subunit-related protein.

Example 5. Construction of a coding sequence of the invention using site-directed mutagenesis

Single stranded DNA of phage pRIT20001, prepared according to Example 1, was purified and submitted to oligonucleotide-driven site-directed mutagenesis using the "Oligonucleotide-Directed in vitro Mutagenesis System" commercialized by Amersham International PLC, Amersham, United Kingdom, code RPN2322, under the conditions recommended by the supplier. The oligonucleotide used to specifically delete the codon TGG coding for the tryptophan residue at the 26th amino acid position of the rS1d gene in pRIT20001 had the sequence 5′ CGTTGTTTCCCGCCGTGAAT 3′. The oligonucleotide used to specifically change the codon TGG coding for the tryptophan residue at the 26th amino acid position of the S1 gene in pRIT20001 had the sequence 5′ CGTTGTTTCCGGTCGCCGTGAAT 3′

The mutant candidates were screened by differential hybridization with the mutagenic oligonucleotides by the technique of Wallace, in Suggs. Developmental Biology Using purified Genes, Ed. D. Brown, N.Y., Academic press. The mutations were then verified by DNA sequencing by the technique of Sanger et al., (1977) supra. One candidate, named pRIT20002 and containing the Trp-26 deletion mutation, was cloned and further analyzed for its ability to express the Trp-26 modified S1 gene as described in Example 6, infra. One candidate, named pRIT20008 and containing the Trp-26 to Thr substitution mutation was cloned and further analyzed for its ability to express the Trp-26 modified S1-related gene as described in Example 6, infra.

Example 6. Expression of a Trp-26 modified rS1d genes in E. coli and analysis of the enzymatic activities of their products

E. coli TG1 cells were infected with pRIT20002 or pRIT20008, both prepared as described in Example 5, and expression of the Trp-26 modified S1 subunit-related genes containing the deletion of the Trp-26 codon TGG or its substitution by codon ACC for Threonine was induced with IPTG as described in Example 2. Cells were then harvested and lysed, the cell lysates fractionated by centrifugation, and the supernatant fractions analyzed for the presence of the mutated rS1d by Western blot and reaction with the anti-S1 monoclonal antibody B2F8, all of the above methods carried out as described in Example 2.

The results were compared to the results obtained in Example 2. The supernatant fractions of cells infected by pRIT20001, pRIT20002 or pRIT20008 contained a protein that reacted with monoclonal antibody B2F8 on Western blots, whereas supernatant fractions of cells infected with M13mp18 did not contain such a protein. Furthermore, the B2F8-reactive proteins in the pRIT20001, pRIT20002 and pRIT20008 supernatants showed the same apparent molecular weight as the partially purified rS1d referred to in Example 3. Based on visual inspection of the Western blots, pRIT20001, pRIT20002 and pRIT20008 expressed roughly similar amounts of

their respective S1 subunit-related proteins.

The pRIT20001, pRIT20002 and pRIT20008 supernatant fractions were compared for their level of NAD-glycohydrolase and ADP-ribosyltransferase activities in the assay systems described in Example 3. Whereas 25 µl of the pRIT20001 supernatant fraction again catalyzed the release of 300 pmoles nicotinamide in a three hour reaction, the pRIT20002 and pRIT20008 supernatant frac tions showed NAD-glycohydrolase activity levels similar to supernatant fractions from cells infected with M13mp18 i.e., substantially no NAD-glycohydrolase activity. Similarly, although the pRIT20001 supernatant fraction was able to catalyze the ADP-ribosylation of the Gi protein, no such activity could be detected with the pRIT20002 supernatant fraction or with the pRIT20008 supernatant fraction. Thus, the S1 subunit related proteins encoded by pRIT20002 and pRIT20008 had substantially inactivated S1 subunit protein enzymatic activities.

Example 7. Antigenic structure of the S1 subunit-related protein encoded by pRIT20002 and pRIT20008

The pRIT20002 and pRIT20008 supernatant fractions, prepared as described in Example 6, were analyzed for their ability to react with monoclonal as well as polyclonal anti-pertussis toxin antisera by Western blots. Polyclonal antibodies to pertussis toxin, obtained from E. Simoen, SmithKline-R.I.T., s.a., Rixensart, Belgium, (SK-RIT), as well as several monoclonal anti-pertussis toxin antibodies, obtained from M. Francotte (SK-RIT), and J.G. Kenimer (Laboratory of Cellular Physiology, Food and Drug Administration, Bethesda, MD), were found to react specifically with the pRIT20002 and pRIT20008 derived S1 subunit related proteins in the crude E. coli cell supernatants. Some of the reacting monoclonal antibodies had good toxin neutralizing and protective activities in mouse challenge systems. This result indicates that at least some protective epitopes are still conserved in the pRIT20002 and pRIT20008 derived proteins, and that the protein has utility in a vaccine against whooping cough.

Example 8. Reconstruction of the complete pertussis toxin operon containing a Trp-26 deletion in the S1 subunit cistron

A 4.7 kilo base pair (kbp) DNA fragment, obtained from T. Cabezon (SK-RIT), containing the complete pertussis toxin operon was inserted into pUC7 which had been previously digested with EcoRI. Such insertion was accomplished according to the method of Maniatis et al. supra. PUC7 is described by J. Vieira & J. Messing, Gene 19:259-268 (1982).

The resulting recombinant plasmid was introduced into E. coli TG1 competent cells according to the method of Maniatis et al., supra. The transformed cells were cloned by the method of Maniatis et al., supra and the clones analyzed by restriction digests of their plasmids by the method of Maniatis et al., supra. One plasmid, named pRIT13070 and containing the entire pertussis toxin operon, was purified and then digested with AccI according to the method of Maniatis et al., supra. The resulting two fragments were separated by polyacrylamide gel electrophoresis (PAGE) and the larger one, was purified and used in the next ligation step.

The replicative form of pRIT20002 was purified and also digested with AccI. The resulting fragments were separated by PAGE and the 300 bp DNA fragment containing the Trp-26 deletion was purified. The 300 bp AccI fragment from pRIT20002 was then ligated into the large AccI fragment from pRIT13070, according to the method of Maniatis et al., supra, and the resulting recombinant plasmid was introduced into E. coli TG1 competent cells by the method of Maniatis et al., supra. After cloning, plasmid pRIT13071 containing the entire pertussis toxin operon was isolated by the method of Maniatis et al., supra. pRIT13071 contains the dele tion of the Trp-26 codon in the S1 cistron, but is otherwise identical to pRIT13070.

The Trp26 modified pertussis toxin operon contained by pRIT13070 can now be used to introduce the modification into the B. pertussis genome by exchange of the resident wild type operon with the modified operon by homologous recombination. Alternatively, the modified operon can also be incorporated in a plasmid which is subsequently transformed into a Bordetella cell or any other appropriate host cell for expression of the protein encoded by the Trp-26 modified operon.

Such modified Bordetella strains will then be used to the coding sequence of this invention and/or other protective antigens for example FHA and/or agglutinogens. These antigens will then be used as vaccines against whooping cough with appropriate adjuvants such as aluminum adjuvants.

Example 9. Parenteral Vaccine preparation

5-25 µg of the protein encoded by pRIT20002 or pRIT20008 (Example 6) is mixed with an aluminum adjuvant such as aluminum hydroxide, to produce a vaccine in a form appropriate for incorporation into a parenteral administration dosage form.

It is appreciated that the invention is not limited to the particular embodiments described above in the Examples. All embodiments of the invention, therefore, are believed to come within the scope of the following claims.

Example 10. Expression of the mutated pertussis toxin gene in Bordetella pertussis

Plasmid pRIT13071, prepared according to the method of Example 8, was digested with restriction enzyme EcoRI, and the entire pertussis toxin operon containing the deletion of the Trp-26 codon in the S1 cistron was isolated as a 4.7 kilobase pair (kbp) DNA fragment by the methods of Maniatis et al., supra. This 4.7 kbp DNA fragment was then inserted into the single EcoRI site of the low copy number plasmid pLAFRII to result in plasmid pRIT13268. Plasmid pLAFRII is equivalent to pLAFRI described by Friedman et al., Gene, 18:289-296

(1982), and was provided by Dr. J.J. Mekalanos, Harvard Medical School, Cambridge, Massachusetts, U.S.A. The recombinant plasmids were transformed into E. coli strain SM10 which was, provided by Dr. S. Stibitz, U.S. Food and Drug Administration, Bethesda, Maryland, U.S.A. pLAFRII carries a tetracyclin resistance gene and therefore SM10 transformants were selected on LB medium (Maniatis et al., supra.) containing 12.5 µg/ml tetracyclin (Maniatis et al., supra.). The tetracyclin resistant transformants were further analyzed by DNA sequencing (Sanger et al., Proc. Natl. Acad. Sci. USA, (1977), supra) for the presence of the mutated pertussis toxin gene. The tetracyclin resistant SM10 clones containing the modified pertussis toxin gene were then conjugated with Bordetella pertussis strain SK39. Strain SK39 is described by Knapp, and Mekalanos, J. Bacteriol., 170, 5059-5066 (1988) and was provided by Dr. J.J. Mekalanos, supra. The SK39 strain contains a Tn5 insertion in the S1 cistron of the pertussis toxin operon in the chromosome. Because of this interruption of the toxin gene, this strain does not synthesize pertussis toxin. Tetracyclin resistant B. pertussis SK39 exconjugates were identified on BC agar (Difco Laboratories, Detroit, Michigan, U.S.A.) supplemented with 250 ml of defibrinated sheep blood per liter (BG medium) and 20 µg/ml tetracyclin. The tetracyclin resistant clones were then grown in the modified Stainer-Scholte broth described by Kloos, et al. in C.R. Manclark and J.C. Hill (eds.), International Symposium on Pertussis, U.S. Department of Health, Education, and Welfare, Washington, D.C., pp. 70-80 (1979). After growth, the cells were separated from the culture medium by centrifugation. Both the cells and the culture medium were then analyzed by Western Blot as described by Locht et al., Infect. Immun. (1987), supra, for the presence of pertussis toxin either as a cell associated form or free in the culture medium. The results indicate that B. pertussis strain SK39 containing the recombinant plasmid does synthesize pertussis toxin and that the toxin is released in the culture supernatant, although significant amounts of pertussis toxin can be found in a cell-associated form. B. pertussis strain SK39 without the recombinant plasmid did not contain detectable amounts of pertussis toxin in either the culture medium or associated to the bacterial cells. It should also be noted that the B. pertussis strain SK39 containing the recombinant plasmid is useful in a whole cell vaccine. Such vaccine is prepared according to conventional techniques.

Using the methods of Examples 8 and 10, but using pRIT20008 instead of pRIT20002, plasmid pRIT13269 was created. pRIT13269 contains the complete pertussis toxin coding sequence but threonine (Thr) is substituted for the tryptophan mutation at amino acid position 26 in the S1 coding sequence.

Example 11. Biological activity of the mutated pertussis toxin

Purified pertussis toxin, purchased from List Biologicals, Campbell, California, U.S.A., as well as culture supernatants from toxin synthesizing B. pertussis strain Tohama I induce morphological changes in Chinese Hamster Ovary (CHO) cells, as described by Hewlett et al., Infect. Immun., 40:1198-1203 (1983). On the other hand, the culture supernatant of B. pertussis strain SK39 containing the recombinant plasmid with the modified pertussis toxin gene induced no detectable morphological changes in the CHO cells, indicating that the deletion of the Trp-26 codon in the S1 subunit cistron of the pertussis toxin gene drastically reduced the biological activity of pertussis toxin. When the activity of the mutant toxin was compared on a more quantitative basis, the mutant appeared at least 500 fold less toxic in this CHO cell assay.

Example 12. Construction of a B. pertussis strain in which the pertussis toxin operon is deleted

To express an entire or truncated B. pertussis toxin S1 subunit amino acid coding sequence which contains a modification of the tryptophan residue at amino acid position 26, it is preferable to use a B. pertussis recipient strain in which the chromosomal toxin gene is deleted, rather than inactivated by point or insertion mutations. Inactivation of the gene through point or insertion mutations does not eliminate DNA sequences that have the potential of recombining in vivo with the DNA coding for the mutated pertussis toxin on a autonomously replicating plasmid. On the other hand, in a pertussis toxin gene deletion mutant, no such recombination can occur. Therefore, the pertussis toxin coding sequences of B. pertussis Tohama I strain, were deleted in the following way. First, the B. pertussis Tohama I strain was plated on BG medium (supra) containing 400 µg/ml streptomycin. Streptomycin resistant strains were then plated on BG medium (supra) oontaining 50 ug/ml nalidixic acid. The B. pertussis Tohama I streptomycin and nalidixic resistant strains were used for the conjugation experiments.

Plasmid pTOX9, described by Black and Falkow, Infect. Immun., 55:2465-2470 (1987), and provided by Dr. W.J. Black, Stanford University Medical School, Stanford, California, U.S.A., contains the pertussis toxin gene on an approximately 10 kbp B. pertussis DNA fragment. pTOX9 was digested with KpnI and BglII restriction enzymes and then with Bal31 exonuclease. After ligation, the deleted pertussis toxin gene was isolated on an approximately 7 kbp ClaI/ClaI DNA fragment from the Bal31 treated pTOX9. All the methods used for the treatment of pTOX9 are described by Maniatis et al. (1982), supra. The 7 kbp DNA fragment was then inserted into the unique HindIII site of plasmid pSORTP1, a derivative of pRTP1 containing a gentamycin-resistent gene described by Stibitz, et al., Gene, 50:133-140 (1986), which was provided by Dr. S. Stibitz, supra. The recombinant plasmid was transformed into E. coli strain SM10 (supra) and analyzed by DNA sequencing to identify the deletion mutation. E. coli SM10 containing the recombinant plasmid with the desired deletion was then conjugated to the streptomycin, nalidixic acid resistant B. pertussis strain Tohama I. The exconjugates containing the recombinant plasmid inserted into the chromosome were identified by their resistance to gentamicin and their sensitivity to streptomycin on BG agar plates (supra). Plasmid pSORTP1 contains the gene for resistance to gentamicin and the gene for the dominant sensitivity to streptomycin, and is not able to

autonomously replicate in B. pertussis. Therefore, gentamicin resistant, streptomycin sensitive B. pertussis strains can only be generated if the recombinant plasmid is inserted in the chromosome by homologous recombination. Such homologous recombination possibly involves the toxin gene flanking regions in the chromosome and the recombinant plasmid. The gentamicin resistant clones were then plated onto BG plates containing 400 µg/ml streptomycin (supra) to select for streptomycin resistant B. pertussis revertants. Some of these revertants appeared because of a second homologous recombination between the chromosome and the inserted plasmid. The resulting streptomycin resistance. strains were analyzed for the loss of gentamicin resistant These clones were then analyzed by Southern blot hybridization, as described by Maniatis et al. (1982), supra, for the loss of the pertussis toxin gene in the chromosome. The resulting B. pertussis strain (hereinafter referred to as "BPRA") is then used for the introduction of plasmids containing the mutated pertussis toxin genes, such as the pLAFRII-derived plasmids described in Example 10 such as pRIT13268. After such plasmids are introduced, the resulting B. pertussis strain BPRA(pRIT13268) is used to produce mutated pertussis toxin. Alternatively, after such plasmids are introduced, the resulting B. pertussis strain is useful in a whole cell vaccine. Such whole cell vaccine is prepared according to conventional techniques well known in the art.

Example 13. Expression in Bordetella pertussis of pertussis toxin genes containing single or double mutations

BPRA, the Bordetella pertussis strain in which the pertussis toxin structural gene was deleted, prepared as described in Example 12, was used as a recipient strain for plasmids derived from pLAFRII. Plasmid pLAFRII is described in Example 10. The pLAFRII-derived plasmids contained the pertussis toxin gene with either single or double mutations. The single mutants contained either a deletion or substitution of Trp-26, or a deletion or substitution of Glu-129 (i.e., the glutamic acid residue at amino acid position 129 in the S1 subunit of the pertussis toxin coding sequence). It is interesting to note that Glu-129 has been previously shown to also be involved in enzymatic activities of pertussis toxin [See, Pizza et al., Proc. Natl. Acad. Sci. USA:85, 7521-7525 (1988)]. The double mutant pLAFRII-derived plasmids contain the deletion of Trp-26 and either the deletion of Glu-129 (plasmid pRIT13270) or the substitution of Glu-129 by aspartic acid (Asp) (plasmid pRIT13271). The genetic manipulations were carried out using classical subcloning techniques as described by Maniatis et al. (1982), supra. Site-directed mutagenesis was performed using the "Oligonucleotide-directed in vitro mutagenesis system" commercialized by Amersham International plc., Amersham United Kingdom, code RPN2322, as described for the Trp-26 mutation exemplified in Example 5. After mutagenesis, all mutants were completely sequenced using the DNA sequencing technique described by Sanger et al., Proc. Natl. Acad. Sci. USA (1977), supra. The pLAFRII-derived plasmids containing the pertussis toxin gene with the different mutations were transformed into E. coli strain SM10, and then were conjugated into the recipient B. pertussis strain by the procedure described in Example 10, with the exception that the B. pertussis recipient strain in this case is BPRA, the strain in which the chromosomal pertussis toxin structural gene has been deleted as described in Example 12. The B. pertussis exconjugants were analyzed by Western blot, as described in Example 10, for the presence of pertussis toxin.

The mutant toxins were then compared to purified pertussis toxin (purchased from List Biological Laboratories, Campbell, California), to the wild type pertussis toxin in the supernatant fraction of B. pertussis Tohama I (supra), and to the supernatant fraction of the strain of B. pertussis in which the chromosomal toxin gene was deleted. Such comparison was based on the biological activities on CHO cells as described in Example 11. It was observed that 3 ng of purified pertussis toxin caused a significant cytotoxic clustering of the CHO cells. These morphological changes were also observed when the culture supernatant of B. pertussis Tohama I was assayed. In contrast, all the mutant toxins in the form of culture supernatant induced only background CHO cell cytotoxicity. On a quantitative basis, all the mutant toxins were estimated to be at least 500 fold less toxic than the wild type pertussis toxin.

The above description fully discloses the invention including preferred embodiments thereof. Modifications and improvements of the embodiments specifically disclosed herein are within the scope of the following claims.

Claims

1. A Bordetella pertussis toxin coding sequence containing an entire or truncated B. pertussis toxin S1 subunit amino acid coding sequence provided that (a) the S1 subunit coding sequence contains a modification of the tryptophan residue at amino acid position 26 (hereinafter "Trp-26"), and (b) the Trp-26 modification results in substantially inactivated S1 enzymatic activity in the protein encoded by the B. pertussis toxin coding sequence but such protein has retained the capacity to be recognized by anti-pertussis toxin antibodies.

2. The B. pertussis toxin coding sequence of Claim 1 wherein said coding sequence comprises the entire Bordetella pertussis toxin operon amino acid coding sequence.

3. The B. pertussis toxin coding sequence of Claim 1 wherein said coding sequence comprises a truncated Bordetella pertussis toxin operon.

4. The B. pertussis toxin coding sequence of Claim 1 wherein said coding sequence comprises only the S1 subunit amino acid coding sequence.

5. The B. pertussis toxin coding sequence of Claim 1 wherein said coding sequence comprises only a truncated portion of the S1 subunit amino acid coding sequence.

6. The B. pertussis toxin coding sequence of Claim 5 wherein said coding sequence comprises only amino acids 2 through 187 of the S1 subunit amino acid coding sequence.

7. The B. pertussis toxin coding sequence of Claim 1 wherein said coding sequence additionally comprises at least one other coding sequence.

8. The B. pertussis toxin coding sequence of Claim 7 which has the following coding sequence:

```
ATG   ACC ATG ATT ACG AAT TCG AGC TCG GTA CCC GGG GAT
(Met) Thr Met Ile Thr Asn Ser Ser Ser Val Pro Gly Asp
          beta-galactosidase    polylinker          2
                                                   S1

CCT CCC GCC ACC GTA...... GTA GCG TCG ATC CTC TAG
Pro Pro Ala Thr Val...... Val Ala Ser Ile Leu Stop
 3   4   5   6   7        184 185 186 187 polylinker
        S1
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9. The B. pertussis toxin coding sequence of Claim 1 wherein said coding sequence additionally comprises a modification of an additional amino acid residue in the S1 subunit coding sequence, provided that the protein encoded by the B. pertussis toxin coding sequence retains the capacity to be recognized by anti-pertussis toxin antibodies.

10. The B. pertussis toxin coding sequence of Claim 9 wherein the additional modification occurs at the glutamic acid residue at amino acid position 129 of the S1 subunit coding sequence (Glu-129); the histidine residue at amino acid position 35 of the S1 subunit coding sequence (His-35); the arginine residue at amino acid position 9 of the S1 subunit coding sequence (Arg-9) or the serine residue at amino acid position 40 of the S1 subunit coding sequence (Ser-40).

11. The B. pertussis toxin coding sequence of Claim 10 wherein the additional modification occurs at the glutamic acid residue at amino acid position 129.

12. A recombinant DNA molecule comprising the Bordetella pertussis toxin coding sequence of Claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11.

13. A recombinant DNA plasmid comprising the recombinant DNA molecule of Claim 12 in operative association with suitable expression control sequences.

14. The recombinant DNA plasmid of Claim 13 which is pRIT20002.

15. The recombinant DNA plasmid of Claim 13 which is pRIT20008.

16. The recombinant DNA plasmid of Claim 13 which is pRIT13070.

17. The recombinant DNA plasmid of Claim 13 which is pRIT13268.

18. The recombinant DNA plasmid of Claim 13 which is pRIT13269.

19. The recombinant DNA plasmid of Claim 13 which is pRIT13270.

20. The recombinant DNA plasmid of Claim 13 which is pRIT13271.

21. A host cell transformed with the plasmid of Claim 13.

22. The host cell of Claim 21 which is BPRA.

23. Protein encoded by the coding sequence of Claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11.

24. A vaccine for stimulating protection against whooping cough wherein such vaccine comprises an immunoprotective and non-toxic quantity of the protein encoded by the coding sequence of Claim 1.

25. A method for innoculating a human against subsequent whooping cough which comprises administering the vaccine of Claim 24 to such human.

26. A process for producing a transformed host cell which comprises transforming a desired host cell with the recombinant DNA plasmid containing the coding sequence of Claim 1.

27. A process for producing the protein encoded by a Bordetella pertussis toxin amino acid coding sequence containing an entire or truncated B. pertussis toxin S1 subunit amino acid coding sequence provided that (a) the S1 subunit coding sequence contains a modification of the tryptophan residue at amino acid position 26 (hereinafter "Trp-26"), and (b) the Trp-26 modification results in substantially inactivated S1 enzymatic activity in the protein encoded by the B. pertussis toxin operon amino acid coding sequence but such protein has retained the capacity to be recognized by anti-pertussis toxin antibodies, wherein such process comprises culturing a host cell transformed with a recombinant DNA plasmid comprising the recombinant DNA coding sequence of Claim 1 in suitable culture medium and isolating the protein so produced.

28. Protein produced by the process of Claim 27.

29. A vaccine for stimulating protection against whooping cough wherein such vaccine comprises an immunoprotective and non-toxic quantity of the protein of Claim 28.

30. A method for innoculating a human against subsequent whooping cough which comprises administering the vaccine of Claim 29 to such human.

31. A process for producing the protein encoded by a Bordetella pertussis toxin amino acid coding sequence containing an entire or truncated B. pertussis toxin S1 subunit amino acid coding sequence provided that (a) the S1 subunit coding sequence contains a modification of the tryptophan residue at amino acid position 26 (hereinafter "Trp-26"), and (b) the Trp-26 modification results in substantially inactivated S1 enzymatic activity in the protein encoded by the B. pertussis toxin operon amino acid coding sequence but such protein has retained the capacity to be recognized by anti-pertussis toxin antibodies, wherein such process comprises (i) treating a Bordetella pertussis toxin amino acid coding sequence, prepared by isolation from native B. pertussis DNA or by synthetic or recombinant DNA techniques, containing an entire or truncated B. pertussis toxin S1 subunit amino acid coding sequence, provided that the S1 subunit coding sequence does not already contain a modification of the tryptophan residue at amino acid position 26, with site-directed mutagenesis, a tryptophan modifying chemical reagent or photooxidation to produce a modification or deletion of the tryptophan residue at amino acid position 26, and transforming a desired host cell with a plasmid containing such resulting coding sequence and culturing such host in suitable culture medium and isolating the protein so produced, (ii) substituting or deleting the tryptophan residue at position 26 of the S1 subunit of a Bordetella pertussis toxin amino acid coding sequence, prepared by isolation from native B. pertussis DNA or by synthetic or recombinant DNA techniques, containing an entire or truncated B. pertussis toxin S1 subunit amino acid coding sequence with another amino acid, and transforming a desired host cell with a plasmid containing such resulting coding sequence and culturing such host in suitable culture medium and isolating the protein so produced, or (iii) preparing such coding sequence by synthetic methods and transforming a desired host cell with a plasmid containing such resulting coding sequence and culturing such host in suitable culture medium and isolating the protein so produced.

32. Protein produced by the process of Claim 31.

33. A vaccine for stimulating protection against whooping cough wherein such vaccine comprises an immunoprotective and non-toxic quantity of the protein of Claim 32.

34. A method for innoculating a human against subsequent whooping cough which comprises administering the vaccine of Claim 33 to such human.

35. A B. pertussis strain which contains an inactivated chromosomal pertussis toxin gene and which additionally comprises the coding sequence of Claim 1.

36. A vaccine for stimulating protection against whooping cough wherein such vaccine comprises an immunoprotective and non-toxic quantity of the strain of Claim 35.

37. A method for innoculating a human against subsequent whooping cough which comprises administering the vaccine of Claim 36 to such human.